Oxygenation of 18-hydroxycorticosterone as the final reaction for aldosterone biosynthesis

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Abstract. Incubation of 18-hydroxycorticosterone with the sonicated mitochondrial preparation of bovine adrenal glomerulosa tissue leads to the production of aldosterone, as measured by radioimmunoassay. The in vitro production of aldosterone from 18-hydroxycorticosterone requires both molecular oxygen and NADPH, and is inhibited by carbon monoxide. Cytochrome P-450 inhibitors such as metrapone, SU 8000, SU 10603, SKF 525A, amphenone B and spironolactone decrease the biosynthesis of aldosterone from 18-hydroxycorticosterone. These results support the conclusion that the final reaction in aldosterone synthesis from 18-hydroxycorticosterone is catalyzed by an oxygenase, but not by 18-hydroxysteroid dehydrogenase. By the same preparation, the production of [3H]aldosterone but not [3H]18-hydroxycorticosterone from [1,2-3H]corticosterone is decreased in a dose-dependent manner by addition of non-radioactive 18-hydroxycorticosterone.

Materials and Methods
Preparation of adrenal mitochondrial fraction and incubation

The mitochondrial fraction of zona glomerulosa tissue of bovine adrenal glands and its sonicated preparation were prepared as previously described (Kojima et al. 1982). Protein concentration was determined with a Bio-Rad protein assay kit, using bovine γ-globulin as the standard.

The incubations were performed utilizing one of two techniques. First, in radioimmunoassay experiments, the incubation mixture consisted of 18-hydroxycorticosterone or corticosterone (Makor Chem. Israel, 100 nmol each per flask), the mitochondrial preparation (40 mg protein per flask) and co-factors (final concentration 240 µM) dissolved in 0.33 M sucrose solution buffered with 10 mM Tris-Cl at pH 7.4. Second, in radiotracer experiment, the incubation mixture consisted of [1,2-³H]corticosterone (Amersham, UK, 5 pmol, 20,000 dpm per flask), 18-hydroxycorticosterone (0 ~ 1 x 10⁻⁴ M), the mitochondrial preparation (40 mg protein per flask) and NADPH (final concentration 240 µM) dissolved in

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the 0.33 M sucrose solution buffered with 10 mm Tris-
HCl at pH 7.4.

In each technique, the steroids were dissolved in etha-
hol and then transferred to the incubation flasks. Two
drops of propylene glycol were added per flask, and then
the ethanol was removed under reduced pressure. In
both radioimmunoassay and radiotracer experiments,
the final volume of the incubation mixture was adjusted
to 5 ml. The mixture was incubated at 37°C for 60 min
in an aerobic atmosphere, unless otherwise stated. Imme-
diately following incubation, the reaction was stopped by
addition of methylenecarboxylate (15 ml) and the mixture
was vigorously shaken to extract steroids.

Radioimmunoassay of aldosterone and
18-hydroxycorticosterone
Immunoreactive 18-hydroxycorticosterone was deter-
mined by the method of Martin et al. (1975). Immu-
noreactive aldosterone was determined by the method
of Ito et al. (1972). The assay performance characteristics
demonstrated inter-assay variations of 12.8% for 18-
hydroxycorticosterone and 10.0% for aldosterone. The
amount of 18-hydroxycorticosterone and aldosterone in
the extract from the mixture which was incubated with-
out the substrates was measured as the control. The net
production of each steroid was calculated by subtracting
the content of the steroids in the control incubation
mixture from the steroid content obtained after incuba-
tion with the substrates. The figures in the tables repre-
sent mean values of triplicates. As the findings of re-
peated experiments under the same design of experi-
ments were consistent, representative results for each
type of study are given in this paper.

Quantitation of aldosterone and 18-hydroxycorticosterone
in radiotracer experiment
Non-radioactive 18-hydroxycorticosterone and aldoste-
one (100 nmol each) were added to the extract as the
carriers. Separation and quantitation of aldosterone and
18-hydroxycorticosterone were carried out as previously
reported (Kojima et al. 1982).

Results

Requirement of co-factor for synthesis of aldosterone
by intact and sonicated mitochondrial preparations
under an aerobic condition
NADP+, NADPH or malate was added to the
mitochondrial preparation with 18-hydroxycortic-
osterone prior to incubation. After the incubation,
the amount of aldosterone was measured by radio-
immunoassay. As shown in Table 1, using intact
mitochondria, malate was effective in stimulating
the formation of aldosterone from 18-hydroxycor-
ticosterone, but either NADPH or NADP+ alone
was ineffective. When the mitochondria were soni-
cated, NADPH but not NADP+ was as effective as
malate in stimulating the aldosterone synthesis.

Table 1.
Effects of co-factors and malate upon production of
aldosterone by intact and sonicated mitochondrial pre-
parations under an aerobic condition.

<table>
<thead>
<tr>
<th>Mitochondria</th>
<th>Co-factor or malate (concentration)</th>
<th>Production of aldosterone (fmol/mg protein for 60 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>Malate (10 mM)</td>
<td>89.4 (100)*</td>
</tr>
<tr>
<td></td>
<td>NADPH (240 µM)</td>
<td>9.7 (11)</td>
</tr>
<tr>
<td></td>
<td>NADP+ (240 µM)</td>
<td>9.8 (11)</td>
</tr>
<tr>
<td>Sonicated</td>
<td>NADPH (240 µM)</td>
<td>96.5 (100)**</td>
</tr>
<tr>
<td></td>
<td>NADP+ (240 µM)</td>
<td>10.6 (11)</td>
</tr>
</tbody>
</table>

* Relative ratio (%) to the yield obtained with malate.
** Relative ratio (%) to the yield obtained with NADPH in case of sonicated mitochondria.

Influence of incubation gas phase
upon formation of aldosterone
from 18-hydroxycorticosterone and corticosterone
To examine possible involvement of cytochrome
P-450 in aldosterone biosynthesis, corticosterone
and 18-hydroxycorticosterone were respectively
incubated with the sonicated mitochondrial frac-
tion in the presence of NADPH under an oxygen-
enriched, or carbon monoxide-replaced, atmos-
phere. When aldosterone was measured by radio-
immunoassay, production of aldosterone from 18-
hydroxycorticosterone was severely reduced in an
atmosphere of 100% carbon monoxide (Table 2). When
oxygen was introduced into the carbon monoxide at 10% (v/v) as the final concentration,
production of aldosterone was somewhat stimu-
lated. In contrast, when the carbon monoxide was
replaced with argon in the same concentration
of oxygen (10%, v/v), production of aldosterone
was markedly enhanced. Similarly, production of
18-hydroxycorticosterone as well as aldosterone
from corticosterone was inhibited by 100% carbon
monoxide, but enhanced by the molecular oxygen
(Table 2).
Table 2.
Effects of incubation atmosphere upon production of aldosterone and 18-hydroxycorticosterone in the presence of NADPH.

<table>
<thead>
<tr>
<th>Gas phase (% v/v)</th>
<th>18-hydroxycorticosterone (substrate)</th>
<th>Corticosterone (substrate)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aldosterone</td>
<td>18-hydroxycorticosterone</td>
</tr>
<tr>
<td>O₂</td>
<td>CO</td>
<td>Ar</td>
</tr>
<tr>
<td>10 90 0</td>
<td>35.1* (36)**</td>
<td>120.6* (43)**</td>
</tr>
<tr>
<td>0 100 90</td>
<td>98.9 (100)</td>
<td>282.4 (100)</td>
</tr>
<tr>
<td>0 100 0</td>
<td>20.7 (21)</td>
<td>108.2 (38)</td>
</tr>
</tbody>
</table>

* fmol/mg protein for 60 min.
** Relative ratio (%) to the yield of steroids obtained in the atmosphere of O₂ and Ar (10:90).

Effect of cytochrome P-450 inhibitors on production of aldosterone and 18-hydroxycorticosterone

The effects of known inhibitors of cytochrome P-450 were demonstrated upon formation of aldosterone and 18-hydroxycorticosterone from their respective precursors, as shown in Table 3. These compounds markedly inhibited aldosterone production from 18-hydroxycorticosterone as well as the formation of aldosterone and 18-hydroxycorticosterone from corticosterone.

Production of 3H-labelled 18-hydroxycorticosterone and aldosterone from [1,2-3H]corticosterone in the presence of non-radioactive 18-hydroxycorticosterone

The sonicated mitochondrial preparation was capable of producing both 3H-labelled 18-hydroxycorticosterone and aldosterone from [1,2-3H]corticosterone. The aldosterone was finally identified by constant specific activities of the crystal obtained through the repeated crystallization: 741, 706, 800, 763 DPM/mg. As shown in Table 4, production of

Table 3.
Influence of cytochrome P-450 inhibitors upon production of aldosterone and 18-hydroxycorticosterone.

<table>
<thead>
<tr>
<th>Inhibitor (5 mM)</th>
<th>Corticosterone (substrate)</th>
<th>18-hydroxycorticosterone (substrate)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>18-hydroxycorticosterone</td>
<td>Aldosterone</td>
</tr>
<tr>
<td>None</td>
<td>340.0* (100)**</td>
<td>208.6* (100)**</td>
</tr>
<tr>
<td>SU 4885</td>
<td>134.2 (39)</td>
<td>63.6 (31)</td>
</tr>
<tr>
<td>SU 8000</td>
<td>108.9 (32)</td>
<td>54.2 (26)</td>
</tr>
<tr>
<td>SU 10603</td>
<td>102.4 (30)</td>
<td>39.6 (19)</td>
</tr>
<tr>
<td>SKF 525A</td>
<td>190.6 (56)</td>
<td>52.1 (25)</td>
</tr>
<tr>
<td>Amphenone B</td>
<td>208.8 (61)</td>
<td>79.3 (38)</td>
</tr>
<tr>
<td>Spironolactone</td>
<td>98.0 (29)</td>
<td>29.2 (14)</td>
</tr>
</tbody>
</table>

* fmol/mg protein for 60 min.
** Relative ratio (%) to the yield of steroids obtained without inhibitors.
Table 4.  
Apparent inhibition of production of [3H]18-hydroxycorticosterone and aldosterone from [1,2-3H]corticosterone by non-radioactive 18-hydroxycorticosterone.

<table>
<thead>
<tr>
<th>Concentration of added non-radioactive 18-hydroxycorticosterone (M)</th>
<th>Production of [3H]18-hydroxycorticosterone</th>
<th>[3H]aldosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1728* (100)**</td>
<td>1048* (100)**</td>
</tr>
<tr>
<td>$1 \times 10^{-10}$</td>
<td>1528 (86)</td>
<td>660 (63)</td>
</tr>
<tr>
<td>$1 \times 10^{-7}$</td>
<td>1812 (102)</td>
<td>260 (25)</td>
</tr>
<tr>
<td>$1 \times 10^{-4}$</td>
<td>1640 (93)</td>
<td>112 (11)</td>
</tr>
</tbody>
</table>

* DPM of radioactive steroid/40 mg protein for 60 min.  
** Relative ratio (%) to the yield of steroids obtained in the absence of non-radioactive 18-hydroxycorticosterone.

The production of aldosterone from the tritiated aldosterone from [1,2-3H]corticosterone was decreased by addition of non-radioactive 18-hydroxycorticosterone in a dose-dependent manner. The result indicates that 18-hydroxycorticosterone is an intermediate for aldosterone synthesis. On the other hand, formation of radioactive 18-hydroxycorticosterone was independent of the amounts of non-radioactive 18-hydroxycorticosterone.

Discussion

By the present experiments, in which the absolute amount of immunoreactive aldosterone produced from 18-hydroxycorticosterone was measured by radioimmunoassay, the enzyme relevant to the final step of aldosterone synthesis required molecular oxygen and NADPH, and was susceptible to carbon monoxide. These results are in agreement with that of our previous work, in which production of [3H]aldosterone from [1,2-3H]18-hydroxycorticosterone was studied (Kojima et al. 1982). On the basis of these findings, the possibility of involvement of ‘18-hydroxysteroid dehydrogenase’ in aldosterone synthesis, as previously postulated, can be excluded. The synthesis of aldosterone from 18-hydroxycorticosterone was suggested to involve cytochrome P-450 as an active constituent of the hydroxylation reaction. SU-compounds, SKF 525A, amphetamine B and spironolactone inhibit enzyme reactions involving cytochrome P-450 for androgen biosynthesis (Inano et al. 1976), but do not affect activity of a hydroxy-steroid dehydrogenase. In the present experiment, these compounds inhibited aldosterone synthesis from 18-hydroxycorticosterone as quantitated by radioimmunoassays (Table 3). This supports our contention that aldosterone is produced from 18-hydroxycorticosterone by cytochrome P-450-linked enzyme system(s), being consistent with the previous data with spironolactone and others (Aupetit et al. 1979, Kojima et al. 1982).

Because of higher yield of aldosterone from corticosterone than that from 18-hydroxycorticosterone (Müller 1980), 18-hydroxycorticosterone has been doubted as the actual intermediate. In the present experiment (Table 4), however, the formation of radioactive aldosterone derived from 1,2-3H-labelled corticosterone was significantly decreased in the presence of non-radioactive 18-hydroxycorticosterone in a dose-dependent manner. The radioactive 18-hydroxycorticosterone synthesized de novo seems to have effectively diluted with exogenous non-radioactive 18-hydroxycorticosterone. The results indicate that 18-hydroxycorticosterone is an intermediate between corticosterone and aldosterone, in agreement with the previous findings (Greengard et al. 1967; Kojima et al. 1982). In this regard, 18-hydroxycorticosterone was converted to aldosterone by a reconstituted system involving cytochrome P-450 purified from bovine adrenal mitochondria (Wada et al. 1984).

In a case of [14C]oestrone synthesis from [14C]androstenedione by human placental microsomes, production of radioactive 19-hydroxyandrostenedione, 19-oxo(or 19,19-dihydroxy)androstenedione and oestrone was competitively and almost equally inhibited by the addition of non-radioactive of 19-oxoandrostenedione, however, production of radioactive oestrone was more severely inhibited than that of 19-oxygenated androgens (Fishman & Goto 1981). From these results, it was concluded that the first and second 19-hydroxylations occurred at the same catalytic site of the aromatase, but the C-10–C-19 cleavage occurred at the other site of the enzyme. Similarly, from the
results of the present tracer experiment (Table 4),
the 18-hydroxylase which is involved in the con-
version of corticosterone to 18-hydroxycorticoste-
one seems different from the enzyme in further
18-hydroxylation of 18-hydroxycorticosterone di-
rected to aldosterone synthesis, because production
of radioactive 18-hydroxycorticosterone from
[3H]corticosterone was not influenced by the addi-
tion of non-radioactive 18-hydroxycorticosterone.
Therefore, the first and second 18-hydroxylases
are postulated as mutually distinct from the pre-
sent results, and from the cases of genetic disorder
of man (Ulick 1973, 1976) and rat (Rapp & Dahl
1976). The first 18-hydroxylase together with 11β-
hydroxylation activity may exist extensively in zona
fasciculata, reticularis and glomerulosa, and is in-
duced by ACTH (Kraemer et al. 1983). The cyto-
chrome P-450 involved in the enzyme system is
responsible for both 11β- and 18-hydroxylation
(Watanuki et al. 1977; Sato et al. 1978). On the
other hand, the second 18-hydroxylase, which is
localized in zona glomerulosa is inhibited by a
prolonged administration of ACTH (Aguilera et
al. 1981), but stimulated by the renin-angiotensin
system (McKenna et al. 1978).

On the basis of our previous and present results,
we propose that the major pathway of aldosterone
biosynthesis is via 18-hydroxycorticosterone and its
conversion by a cytochrome P-450-linked enzyme
system (an oxygenase) to 18,18-dihydroxycorticoste-
one which is finally transformed to aldosterone
by non-enzymatic dehydration (Fig. 1).

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